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Loss of Fibrinogen Rescues Mice from the Pleiotropic Effects of Plasminogen Deficiency

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Summary

Plasmin(ogen) is an extracellular serine protease implicated in the activation of latent growth factors and procollagenase, degradation of extracellular matrix components, and fibrin clearance. Plasminogen (Plg) deficiency in mice results in high mortality, wasting, spontaneous gastrointestinal ulceration, rectal prolapse, and severe thrombosis. Furthermore, Plg-deficient mice display delayed wound healing following skin injury, a defect partly related to impaired keratinocyte migration. We generated mice deficient in Plg and fibrinogen (Fib) and show that removal of fibrin(ogen) from the extracellular environment alleviates the diverse spontaneous pathologies previously associated with Plg deficiency and corrects healing times. Mice deficient in Plg and Fib are phenotypically indistinguishable from Fib-deficient mice. These data suggest that the fundamental and possibly only essential physiological role of Plg is fibrinolysis.

Introduction

The proteolytic conversion of Plg to the active serine protease plasmin is regulated by a complex system of proteins that includes urokinase-type Plg activator (uPA), tissue-type Plg activator (tPA), and Plg and PA-specific receptors and inhibitors (Danø et al., 1985; Saksela and Rifkin, 1988; Vassalli et al., 1991; Collen and Lijnen, 1994; Danø et al., 1994). Expression of components of the PA system, and thus the production of plasmin, is sensitive to a variety of growth factors, cytokines, and hormones, and appears to be intricately regulated in vivo during development and in adult life (Saksela and Rifkin, 1988; Danø et al., 1985; Danø et al., 1994). Plg activation has been documented in a wide variety of physiological processes involving extracellular-matrix degradation, tissue remodeling and cell migration, including ovulation, trophoblast invasion, embryonic development, neuronal cell migration, inflammation, angiogenesis, and wound healing (Strickland et al., 1976; Bode and Dziadek 1979; Valinsky and Reich, 1981; Marotti et al., 1982; Pepper et al., 1987; Moscatelli and Rifkin, 1988; Sappino et al., 1989; Sappino et al., 1991; Rømer et al., 1994). Plg activation has also been associated with several pathological processes, most notably tumor cell invasion and atherosclerosis (Danø et al., 1985, 1994; Grainger et al., 1994).

Plasmin efficiently degrades fibrin and has a well-established role in fibrinolysis (Collen and Lijnen, 1994). However, the biochemical function(s) of plasmin within the context of general extracellular-matrix dissolution, tissue remodeling, and cell migration is less defined, but different roles have been proposed. Plasmin has a relatively broad substrate specificity and is known to degrade several common extracellular-matrix glycoproteins in vitro (Saksela and Rifkin, 1988; Vassalli et al., 1991). In addition, Plg activation has been proposed to lead to matrix degradation indirectly via activation of the matrix metalloproteases (Werb et al., 1977). Furthermore, plasmin can activate several latent growth factors in vitro, including latent transforming growth factor β and latent basic fibroblast growth factor, and these activities have been proposed to be of crucial importance for cell migration and tissue remodeling in vivo (Brunner et al., 1991; Naldini et al., 1992; Odekon et al., 1994). Finally, a noncatalytic fragment of Plg, angiostatin, has recently been shown to be a potent inhibitor of angiogenesis (O'Reilly et al., 1994).

The recent generation of mice with deficiencies in Plg, PA, uPA receptor (uPAR), and PA inhibitor-1 (Carmeliet et al., 1994a, 1994b; Bugge et al., 1995a, 1995b, 1996; Ploplis et al., 1995; Dewerchin et al., 1996) has provided an extraordinary opportunity to define in greater detail the role(s) of the Plg-activation system in vivo. Remarkably, mice with each of these deficits, even in combination, were born normal in appearance, survived to adulthood, and produced offspring. Thus, the major components of the PA-Plg system are not strictly required for development, growth, and reproduction. Nevertheless, the consequences of either PA or Plg deficiency are severe and include high mortality, wasting, spontaneous ulcerations throughout the gastrointestinal tract, widespread organ damage, rectal lesions, spontaneous skin ulcerations (Carmeliet et al., 1994a; Bugge et al., 1995a; Ploplis et al., 1995), reduced ovulation efficiency (Leonardson et al., 1995), impaired mammary-gland involution (unpublished data), and susceptibility to bacterial infections (Gyetko et al., 1996). Moreover, when challenged by surgical skin injury, both Plg- and PA-deficient mice took far longer to heal than control animals and displayed a major impediment in keratinocyte migration over wound surfaces (Bugge et al., 1996; Rømer et al., 1996). These later findings are particularly notable in that they clearly establish the importance of PA/Plg in tissue remodeling and cell migration in vivo.

The critical issue of what constitutes the primary mechanism(s) responsible for spontaneous-lesion formation and delayed wound repair in PA/Plg-deficient mice could not be fully resolved in the initial studies of these animals. The diverse spectrum of phenotypes observed in the Plg-deficient animals could be viewed

Table 1. Hematological Parameters in Single and Double-Deficient Mice[a]

	Control	$Plg^{-/-}$	$Fib^{-/-}$	$Plg^{-/-}/Fib^{-/-}$
White blood cells ($\times 10^9$/liter)	4.2 ± 0.7	5.3 ± 4.0	4.2 ± 1.3	3.8 ± 1.3
Red blood cells ($\times 10^{12}$/liter)	9.2 ± 1.2	8.6 ± 0.6	8.3 ± 0.4	8.5 ± 0.4
Hemoglobin (g/%)	14.9 ± 1.4	12.9 ± 0.7	13.0 ± 0.9	13.0 ± 0.7
Hematocrit (%)	47.2 ± 4.0	41.7 ± 1.2	42.0 ± 2.6	41.8 ± 1.9
Mean corpuscular volume (fl)	51.5 ± 2.2	48.7 ± 1.9	50.3 ± 1.7	49.2 ± 1.2
Platelets ($\times 10^9$/liter)	827 ± 141	1263 ± 996	769 ± 209	881 ± 267

[a] Data presented as mean ± standard deviation of five control, three $Plg^{-/-}$, five $Fib^{-/-}$, and seven $Plg^{-/-}/Fib^{-/-}$ mice.

as compatible with functional deficits in virtually any of the processes previously associated with plasmin-mediated proteolysis (e.g., growth-factor and protease activation, general matrix turnover, and fibrin clearance). However, a consistent feature of the varied pathologies associated with Plg or PA deficiency is vascular- and extravascular-fibrin(ogen) deposits within affected organs and tissues (Carmeliet et al., 1994a; Bugge et al., 1995a; Ploplis et al., 1995; Rømer et al., 1996). To specifically address the role of impaired fibrinolysis in the pleiotropic effects of Plg deficiency, we generated mice deficient in both Plg and Fib. We report that Fib deficiency rescues mice from the spontaneous pathologies known to befall Plg-deficient mice, suggesting that fibrinolysis is the only essential physiological role of Plg.

Results

Generation of Mice with Combined Plg and Fib Deficiency

Interbreeding of previously described mouse lines carrying disrupted Plg (Bugge et al., 1995a) and Fib (Suh et al., 1995) genes resulted in the generation of mice with both single ($Plg^{-/-}$, $Fib^{-/-}$) and combined ($Plg^{-/-}/Fib^{-/-}$) deficiencies in Plg and Fib. Consistent with the Mendelian inheritance pattern established previously for the mutant Plg and Fib genes (Bugge et al., 1995a; Suh et al., 1995), crosses between mice carrying both mutant Plg and Fib alleles resulted in the birth of $Plg^{-/-}$, $Fib^{-/-}$, and $Plg^{-/-}/Fib^{-/-}$ progeny in approximately the ratio expected, based on unlinked genes (data not shown). Mice with each of these genotypes appeared normal at birth and generally survived to adulthood (see below). However, shortly after birth, a small fraction of neonates displayed peritoneal bleeding from which they generally recovered, a known feature of Fib-deficient mice (Suh et al., 1995). Aside from the lack of clotting function and fibrinolytic potential documented previously, we found no impact of the single or combined deficiencies on the general hematological profile of the mice (Table 1). Specifically, no differences were found between animals with regard to white blood cell, platelet, and red blood cell counts, hemoglobin, hematocrit, and mean corpuscular volume (Table 1).

Fib Deficiency Alleviates Wasting of Plg-Deficient Mice

To examine the role of single and combined deficiencies in overall growth and survival of the mice, we followed the fate of a prospective cohort of mice initially consisting of 44 control mice, 25 $Plg^{-/-}$ mice, 16 $Fib^{-/-}$ mice, and 23 $Plg^{-/-}/Fib^{-/-}$ mice. $Plg^{-/-}$ mice had essentially normal weight gain until 2 months of age (Figure 1B). Thereafter, a progressive weight loss was observed, which became severe after 4 months. The weight of $Plg^{-/-}$ mice 6 months or older was uniformly less than two-thirds that of control mice (Figures 1A and 1B). In contrast, mice lacking only Fib exhibited normal weight gain as adolescents and no wasting phenomenon as adults, even when followed for more than 12 months (Figures 1A and 1B). Remarkably, in the same genetic background, $Plg^{-/-}/Fib^{-/-}$ mice, despite their lifelong lack of Plg, exhibited the same excellent growth properties as those of control and $Fib^{-/-}$ mice (Figures 1A and 1B). Indeed, no signs of weight loss or wasting were observed in any of more than 20 $Plg^{-/-}/Fib^{-/-}$ mice that were followed for more than a year. This dramatic rescue from the severe-wasting syndrome normally associated with Plg deficiency provided a first indication that general ill health in young $Plg^{-/-}$ mice depended on fibrin(ogen).

Fib Deficiency Reduces Morbidity and Increases Life Expectancy of Plg-Deficient Mice

$Plg^{-/-}$ mice exhibited very high morbidity and mortality (Figure 2 and Table 2). The median survival time of the prospectively established cohort of $Plg^{-/-}$ mice was 176 days, and all these $Plg^{-/-}$ mice died or had to be euthanized by 301 days. Of the $Plg^{-/-}$ mice, 40% died spontaneously. Apart from one mouse with a massive hemorrhage in the chest cavity, subsequent gross necroscopic analysis of $Plg^{-/-}$ mice that had died spontaneously revealed no obvious cause of death. The mice appeared to maintain an appetite until the time of death, as evidenced by the filled stomachs and intestines of all $Plg^{-/-}$ mice analyzed. Of the $Plg^{-/-}$ mice, 52% developed spontaneous rectal ulcerations, which over a period of a few weeks progressed into frank rectal prolapse (Table 2). Mice with ulcerating rectal prolapse had signs of serious distress that necessitated euthanasia.

Although at risk for spontaneous catastrophic hemorrhaging, $Fib^{-/-}$ mice generally exhibit good health in the absence of overt challenges such as injury or pregnancy (Suh et al., 1995). The penetrance of spontaneous fatal bleeding events depends highly on the genetic background of the mice (Suh et al., 1995). In the mixed genetic background in this study (129/CF-1/Black Swiss), unchallenged $Fib^{-/-}$ mice exhibited very modest phenotypic consequences, much like that described earlier for $Fib^{-/-}$ mice in an inbred C57Bl/6 genetic background (Suh et al., 1995). The survival profile of the cohort of

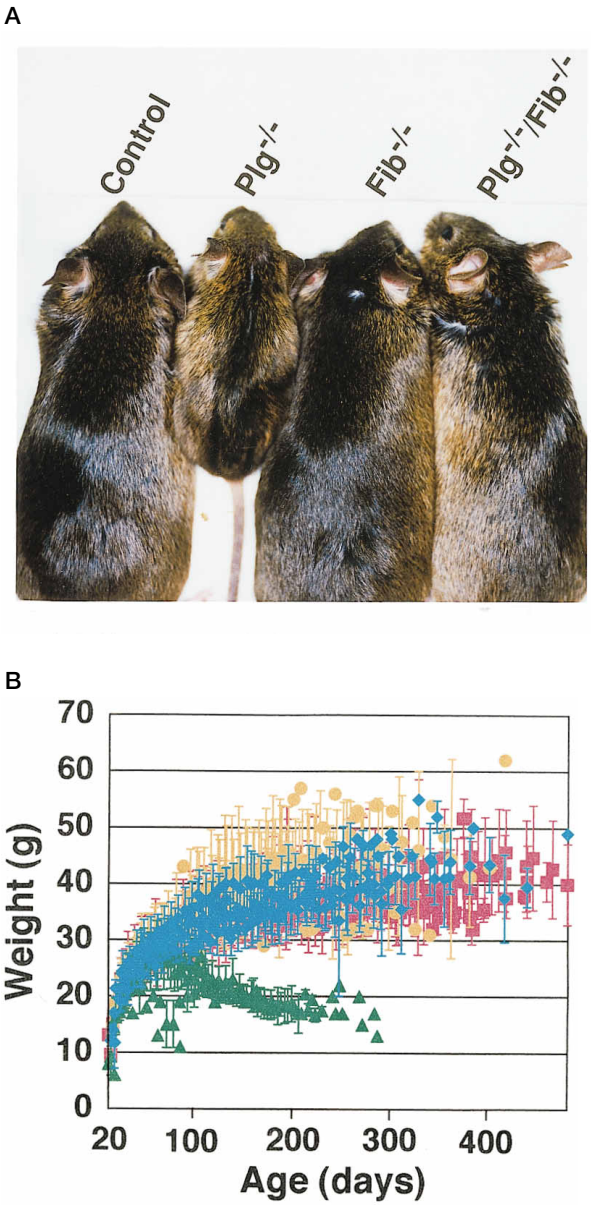


Figure 1. Fib Deficiency Rescues Plg$^{-/-}$ Mice from the Wasting Syndrome

(A) Typical appearance of a control mouse, a Plg$^{-/-}$ mouse, a Fib$^{-/-}$ mouse, and a Plg$^{-/-}$/Fib$^{-/-}$ mouse at seven months of age. Note the severe wasting of the Plg$^{-/-}$ mouse, whereas the appearance of the Plg$^{-/-}$/Fib$^{-/-}$ mouse is indistinguishable from the control and Fib$^{-/-}$ mice.

(B) Plot of weight versus age of control mice (blue diamonds), Fib$^{-/-}$ mice (red squares), Plg$^{-/-}$ mice (green triangles), and Plg$^{-/-}$/Fib$^{-/-}$ mice (gold circles). The weights of a cohort initially consisting of 44 control mice, 25 Plg$^{-/-}$ mice, 16 Fib$^{-/-}$ mice, and 23 Plg$^{-/-}$/Fib$^{-/-}$ mice were measured at 1- to 2-week intervals. Bars indicate the standard deviation. Due to the high mortality associated with Plg deficiency, the number of Plg$^{-/-}$ mice included in the weight measurements decreased over time as follows: days 20–100, 25 decreasing to 19 mice; days 101–200, 19 decreasing to 8 mice; days 201–300, 8 decreasing to 1 mouse.

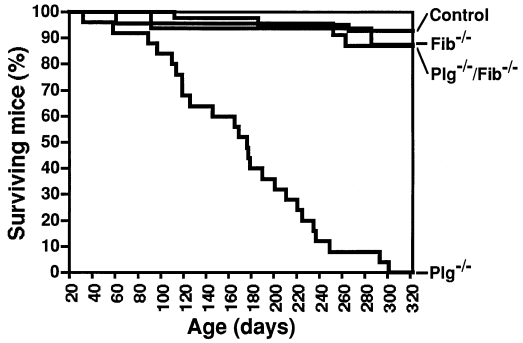


Figure 2. Fib Deficiency Rescues Plg$^{-/-}$ Mice from High Mortality

Survival data are shown for a prospective cohort of 44 control mice, 25 Plg$^{-/-}$ mice, 16 Fib$^{-/-}$ mice, and 23 Plg$^{-/-}$/Fib$^{-/-}$ mice that could be followed for at least 320 days. See Table 2 for details on morbidity and mortality of the mice.

Fib$^{-/-}$ mice followed in this study was similar to that of control mice (Figure 2 and Table 2). Of the 16 Fib$^{-/-}$ mice that could be followed for almost 1 year, only 2 (13%) died, a fraction comparable with that in the cohort of control mice followed in parallel (2 of 44 [5%] died within the 320 day observation period). The excellent survival characteristics of Fib$^{-/-}$ mice made it possible to determine whether fibrin(ogen) deficiency was a benefit to the general survival of Plg$^{-/-}$ mice. The survival profile of Plg$^{-/-}$/Fib$^{-/-}$ mice showed that a lack of fibrin(ogen) rescued animals from the early morbidity and premature death associated with Plg deficiency (Figure 2 and Table 2). The survival characteristics of Plg$^{-/-}$/Fib$^{-/-}$ mice were comparable with those of Fib$^{-/-}$ and control mice, with only 2 (9%) of the cohort of 23 mice becoming terminally ill within a 320 day observation period; 12 Plg$^{-/-}$/Fib$^{-/-}$ animals have been followed for more than 400 days without any overt signs of disease. Parenthetically, it should be noted that Fib$^{-/-}$ and Plg$^{-/-}$/Fib$^{-/-}$ females were also phenotypically similar (and distinct from Plg$^{-/-}$ and control mice) with regard to their inability to survive pregnancy beyond about 10 days (Suh et al., 1995 and data not shown). Taken together, these data suggest that the absence of fibrin(ogen) in Plg$^{-/-}$ mice dramatically increases survival but leaves the mice with the same health and reproductive liabilities associated with a total lack of clotting function (also see below). The main threat to the health and life expectancy of Plg$^{-/-}$/Fib$^{-/-}$ mice appears to be the risk of spontaneous or injury-induced hemorrhaging, an inherent feature of Fib deficiency.

Pathology Associated with Plg Deficiency Is Eliminated by Fib Deficiency

Detailed histological examination of tissues was performed on 13 Plg$^{-/-}$ mice, including 8 young animals (58–110 days old) and 4 older animals (175–211 days old). Of the 13 Plg$^{-/-}$ mice examined, 5 were sacrificed due to rectal prolapse, 3 were apparently healthy young mice without signs of wasting, 4 were older mice with wasting, and 1 mouse was analyzed after dying spontaneously at 110 days (Table 3). All Plg$^{-/-}$ mice analyzed displayed a number of pathological features documented earlier in Plg$^{-/-}$ mice with a 129-Black Swiss

Table 2. Morbidity and Mortality of Single- and Double-Deficient Mice

Group	No. Mice in Cohort	No. Mice Dying Within a 320-Day Observation Period	Cause of Death/Euthanasia	No. Mice	Day of Death/ Euthanasia (Median)
Control	44	2 (5%)	Spontaneous death. No analysis.	1 (2%)	266
			Euthanized due to extensive skin ulceration.	1 (2%)	186
$Plg^{-/-}$	25	25 (100%)	Euthanized due to an ulcerating rectal prolapse with obvious distress to animal.	13 (52%)	89–235 (165)
			Spontaneous death. Absent or inconclusive necropsy.	10 (40%)	32–301 (190)
			Massive hemorrhage of unidentified origin in chest cavity.	1 (4%)	58
			Euthanized due to severe wasting.	1 (4%)	190
$Fib^{-/-}$	16	2 (13%)	Spontaneous death. No analysis.	2 (13%)	92, 286
$Plg^{-/-}/Fib^{-/-}$	23	2 (9%)	Euthanized due to suspected terminal hemorrhaging.[a]	2 (9%)	61, 252

[a] Appearance of animals indicated distress due to hemorrhaging. Gross examination of one mouse showed signs of an internal hemorrhage of undetermined origin. The other mouse suffered resumed bleeding from the site of the tail biopsy.

genetic background (Bugge et al., 1995a). A uniform feature of the $Plg^{-/-}$ mice was multiple lesions scattered throughout the liver, characterized by sinusoidal deposits of fibrillar material (Figure 3A). These fibrillar deposits were shown to be rich in fibrin(ogen), based on immunostaining (data not shown; also see Bugge et al., 1995a). The lesions displayed various degrees of organization with infiltration of spindle cells, possibly macrophages, and fibroblasts. Entrapment of necrotic hepatocytes within the fibrillar material was a common feature in the lesions. Anal-rectal ulceration was another prominent feature of Plg deficiency. Rectal ulceration was histologically documented in all five mice with rectal prolapse and in four of six mice without grossly apparent rectal prolapse. The lesions were characterized by reactive hyperplasia and surface exudate (Figure 3C).

The stomach, another part of the gastrointestinal tract, was highly prone to spontaneous ulceration in the $Plg^{-/-}$ mice (Table 3 and Figure 3E). One stomach ulcer or more was observed in 10 of the 13 mice by inspection of a single histological section from each mouse; the 3 $Plg^{-/-}$ mice without histologically detectable stomach ulcers were among the youngest analyzed. The ulcers in the $Plg^{-/-}$ mice had a characteristic morphology (Figure 3E), with a necrotic surface epithelium covered by exudate, necrotic underlying tissue with diffuse distribution of fibrillar material, reactively altered hyperplasia of adjacent epithelium, and occasional vascular occlusions in the lamina propria. Careful macroscopic examination of the stomachs of two older $Plg^{-/-}$ mice revealed multiple white foci compatible with the histologically detected ulcers (data not shown).

Histological inspection of single sections from other parts of the gastrointestinal tract of the $Plg^{-/-}$ mice revealed colonic ulcers in three 110- to 169-day-old mice and a duodenal ulcer in one 79-day-old mouse. Morphology of the colonic and duodenal ulcers was very similar to that of the stomach ulcers (data not shown). Pathological changes were also frequently detected in the urogenital and respiratory tracts. Pulmonary lesions with a variable degree of cellular organization (Figure 3G) were detected in single sections of five of the mice; similar lesions previously were shown to stain intensely using a fibrin(ogen)-specific antibody (Bugge et al., 1995a). Tracheal fibrin-rich lesions were found in single sections from four $Plg^{-/-}$ mice, in one case associated with necrosis (Table 3, and data not shown). Moreover, of seven $Plg^{-/-}$ virgin females examined, vaginal lesions were documented in four (Table 3). A variety of other pathologies, possibly related to Plg deficiency, were also found in individual $Plg^{-/-}$ mice (Table 3, and data not shown).

Consistent with the remarkably strong growth and survival characteristics of $Plg^{-/-}/Fib^{-/-}$ mice, none of the microscopic abnormalities specifically recognized in $Plg^{-/-}$ animals were documented in the 17 Plg-deficient mice that also lacked fibrin(ogen) (Figure 3 and Table 3). This group of $Plg^{-/-}/Fib^{-/-}$ mice included 14 44- to 110-day-old and 3 175- to 252-day-old mice that were siblings of the histologically analyzed $Plg^{-/-}$ mice. Of these $Plg^{-/-}/Fib^{-/-}$ mice, 15 were randomly selected, apparently healthy animals, and 2 were preterminal animals sacrificed with suspected internal hemorrhaging (see above). Pathology in the $Plg^{-/-}/Fib^{-/-}$ mice was scarce and similar to that of nine $Fib^{-/-}$ mice analyzed in parallel (Table 3). The most prominent feature was sporadic necrotic liver foci (single hepatocyte dropout), a finding in 7 of the 17 $Plg^{-/-}/Fib^{-/-}$ mice and 3 of the 9 $Fib^{-/-}$ mice. The etiology of the single hepatocyte dropout was not evident, but Fib deficiency appears to contribute, as similar lesions were not detected in the livers of the 10 control mice analyzed in parallel (Table 3). One $Plg^{-/-}/Fib^{-/-}$ mouse had an organizing subcapsular hematoma typical of $Fib^{-/-}$ mice (Suh et al., 1995). Other pathologies in the two groups of mice that were not

Table 3. Histological Findings in Single- and Double-Deficient Mice

Group	No. mice	Age (Days) Median; Range	Pathology	Frequency[a]
Control	10	93; 37-186	Skin ulceration	1 (10%)
$Plg^{-/-}$	13	110; 58-211	Liver lesions	13 (100%)
			Rectal ulceration	10 (77%)
			Glandular stomach ulcer[b]	9 (70%)
			Vaginal lesions[c]	4 (57%)
			Pulmonary lesions	5 (39%)
			Squamous stomach ulcer[b]	3 (23%)
			Tracheal thrombi	3 (23%)
			Colonic ulcer	3 (24%)
			Cervicitis[c, d]	1 (14%)
			Tracheal necrosis	1 (8%)
			Larynx thrombi	1 (8%)
			Kidney thrombi	1 (8%)
			Esophageal ulcer	1 (8%)
			Colitis	1 (8%)
$Fib^{-/-}$	9	79; 37-109	Necrotic liver foci	3 (33%)
			Pancreatic cyst	1 (11%)
			Squamous stomach hemorrhage	1 (11%)
$Plg^{-/-}/Fib^{-/-}$	17	83; 44-252	Necrotic liver foci	7 (41%)
			Hemorrhagic organized liver lesion	1 (6%)
			Dystrophic calcification in liver	1 (6%)
			Dystrophic calcification in duodenum	1 (6%)

[a] The data were collated from the analysis of one to two routine sections of organs from each mouse. The sections only cover a fraction of any organ or tissue. The frequencies of the occurrence of the pathologies in the groups of mice are therefore likely to represent a significant understatement.

[b] Macroscopic inspection of the stomach of two older $Plg^{-/-}$ mice revealed 10–20 white foci, likely to be identical to the histologically detected ulcers.

[c] Four control, two $Fib^{-/-}$, seven $Plg^{-/-}$, and six $Plg^{-/-}/Fib^{-/-}$ females were analyzed; all were virgins.

[d] Cervicitis was sporadically detected in the mouse colony and appears to be unrelated to the genotypes of the mice.

found in the control mice included hemorrhaging into the squamous part of the stomach of one $Fib^{-/-}$ mouse, a pancreatic cyst in one $Fib^{-/-}$ mouse, and dystrophic calcification in the liver and duodenum of individual $Plg^{-/-}/Fib^{-/-}$ mice. Therefore, fibrin(ogen) deficiency rescues $Plg^{-/-}$ mice from the widespread tissue damage specifically associated with lack of Plg, but $Plg^{-/-}/Fib^{-/-}$ mice maintain many or all of the pathological features of mice lacking Fib alone.

Rescue of Wound Healing in Plg-Deficient Mice

We have previously shown that healing of incisional skin wounds is severely impaired in $Plg^{-/-}$ mice (Rømer et al., 1996). One major defect in $Plg^{-/-}$ mice appears to be decreased keratinocyte migration from the wound edges leading to delayed reepithelialization (Rømer et al., 1996). The fact that $Fib^{-/-}$ mice can tolerate a full-thickness skin incision without excessive bleeding (data not shown), and that healing time of skin wounds in $Fib^{-/-}$ mice is similar to that of control mice (Figure 4, and data not shown), provided an opportunity to establish whether a complex pathophysiological process, wound healing, was also corrected in $Plg^{-/-}$ mice by the absence of fibrin(ogen). Incisional skin wounds were made in young $Plg^{-/-}/Fib^{-/-}$, $Plg^{-/-}$, $Fib^{-/-}$, and control mice, and the appearance of the wounds and the progress in the healing processes were followed by macroscopic inspection (Figures 4A and 4B). The wounds were initially spindle-shaped with well-separated edges and covered with a dehydrated wound crust or scab the day after surgery. In control mice, this scab was lost after about 10 days, revealing a thin residual defect. These mice were scored as healed from days 11–13 after wounding, based on the macroscopic closure of the incision interface and restoration of epithelial covering (Figure 4B). As expected from our earlier findings (Rømer et al., 1996), wound healing in $Plg^{-/-}$ mice was severely impaired (Figures 4A and 4B). The scab was retained longer, and when lost, revealed a large, gaping wound field lacking epidermal covering. This wound field eventually shrank, and four of five wounds from the $Plg^{-/-}$ mice were macroscopically scored as healed from days 19–39 (Figure 4B). In contrast, the progress of wound healing in $Plg^{-/-}/Fib^{-/-}$ and $Fib^{-/-}$ mice was similar to that observed in control mice (Figure 4 and see below). However, there were slight macroscopic differences. For example, although bleeding was modest in all groups following surgery, the $Plg^{-/-}/Fib^{-/-}$ and $Fib^{-/-}$ mice clearly differed from control mice by the presence of distinctly larger deposits of dried blood at the wound margins. In addition, the scabs of $Plg^{-/-}/Fib^{-/-}$ and $Fib^{-/-}$ mice, presumably consisting of dried blood and tissue exudate, appeared initially less stable than in control animals, and resumed bleeding was occasionally apparent in the first few days after surgery. When this scab was lost after about 10 days, it revealed a thin residual defect similar to that of the wounds in control mice. The wounds from $Plg^{-/-}/Fib^{-/-}$ and $Fib^{-/-}$ mice were scored as healed from

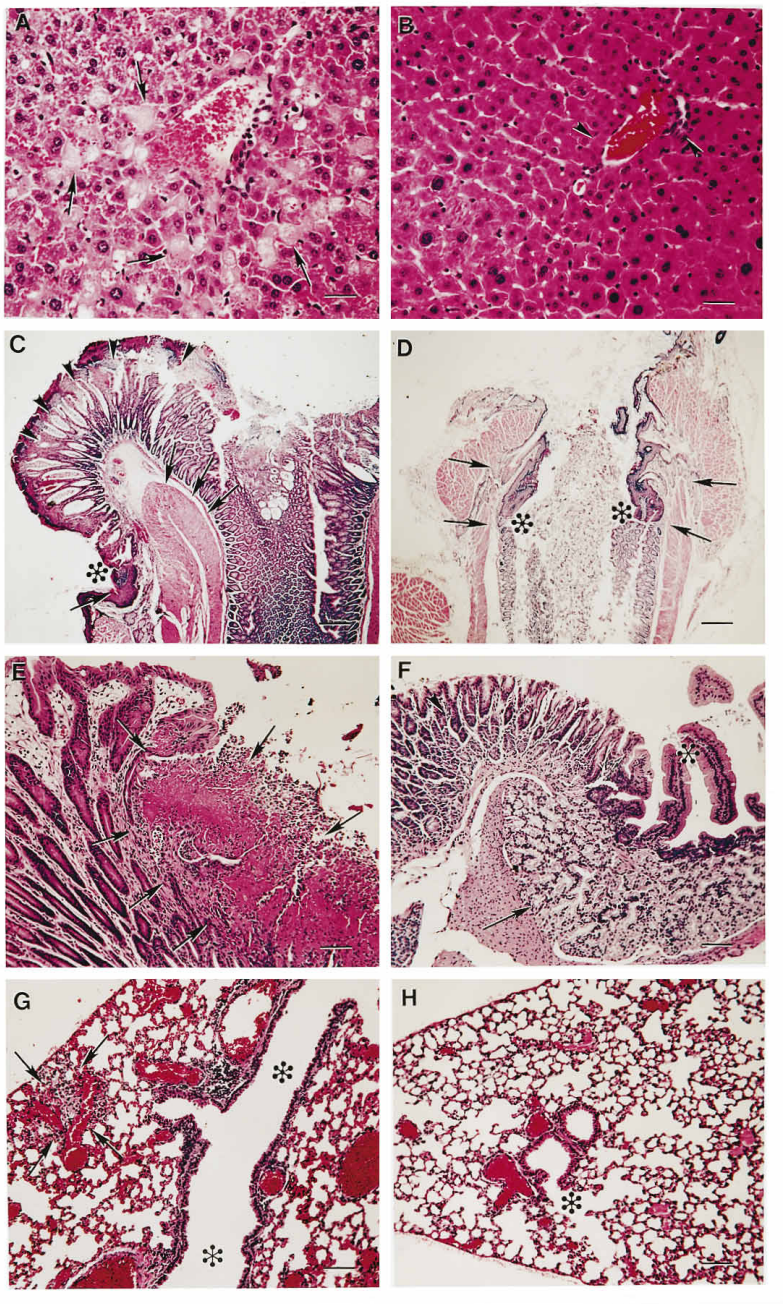


Figure 3. Alleviation of Organ Pathology in $Plg^{-/-}$ Mice by Fib Deficiency

Representative histological sections of the liver (A and B), rectum (C and D), glandular stomach (E and F), and lung (G and H) of $Plg^{-/-}$ mice (A, C, E, and G) and $Plg^{-/-}/Fib^{-/-}$ mice (B, D, F, and H).

(A) Pathological changes in the liver of a 14-week-old $Plg^{-/-}$ mouse. Arrows indicate necrotic hepatocytes trapped in fibrin-rich patches.

(B) Section of a histologically normal liver from a 16-week-old $Plg^{-/-}/Fib^{-/-}$ mouse. Arrowheads delineate normal portal-tract structures. No necrotic hepatocytes are observed.

(C) Longitudinal section of the rectum of a 16-week-old $Plg^{-/-}$ mouse with rectal prolapse, showing extensive ulceration of prolapsed mucosa with surface exudate and reactive hyperplasia (arrowheads). Triple arrows indicate muscular layers prolapsed past the squamocolumnar junction (asterisk).

(D) Longitudinal section of the rectum of a 13-week-old $Plg^{-/-}/Fib^{-/-}$ mouse. The two sets of double arrows indicate normal alignment of anal structures at the squamocolumnar junction (asterisks).

(E) Ulceration of the glandular portion of the stomach (arrows) of a 24-week-old $Plg^{-/-}$ mouse.

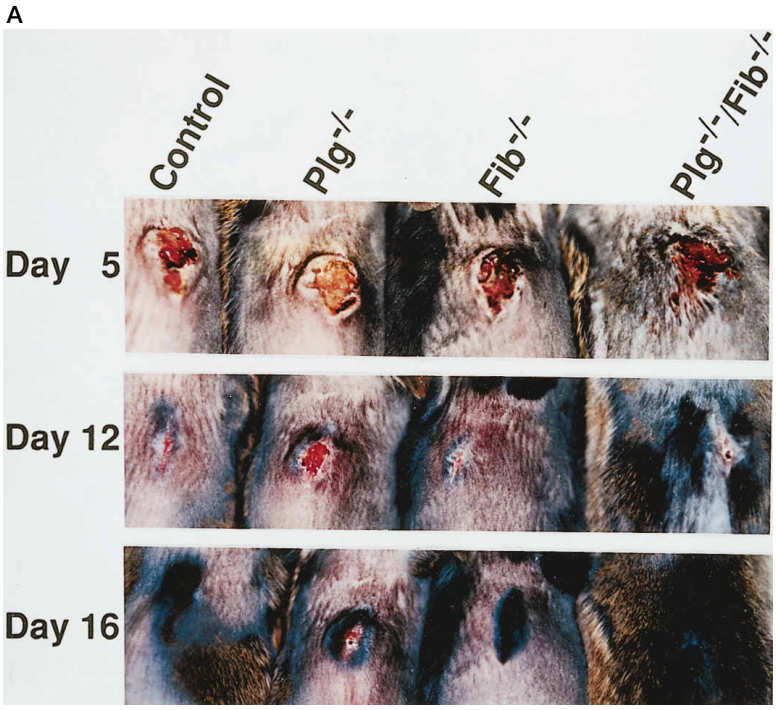
(F) Section of histologically normal glandular stomach and proximal duodenum of a 9-week-old $Plg^{-/-}/Fib^{-/-}$ mouse. The arrowhead indicates the glandular stomach, the arrow indicates Brunners glands underlying the duodenum, and the asterisk indicates duodenal villi.

(G) Area of organization (arrows) in the lung of a 17-week-old $Plg^{-/-}$ mouse. The asterisks indicate a bronchiole.

(H) Corresponding section of a histologically normal lung of a 12-week-old $Plg^{-/-}/Fib^{-/-}$ mouse. The asterisk indicates a terminal bronchiole. Magnification bars in A and B = 35 μm; C and D = 350 μm; E–H = 100 μm.

days 11–15 and days 12–14, respectively (Figure 4B). Thus, viewed macroscopically, the absence of Fib restores the overall rate of wound healing in $Plg^{-/-}$ mice to normal.

To view the healing process microscopically, we collected wound tissues from three $Plg^{-/-}/Fib^{-/-}$, $Plg^{-/-}$, $Fib^{-/-}$, and control mice at each of three time points after incision: days 3, 10, and 17. At day 3, the beginning of an outgrowth of a wedge of keratinocytes could be observed at the margins of all wounds. At this time, no major differences were found between the wounds in the mice of different genotypes (data not shown). However, at days 10 and 17, dramatic differences were observed. By days 10 (data not shown) and 17 (see representative data in Figure 5), the keratinocyte outgrowths extending from each wound edge had met and fused to cover the wounds of control, $Fib^{-/-}$, and $Plg^{-/-}/Fib^{-/-}$ mice completely, regardless of the section inspected. In contrast, the fronts of migrating keratinocytes in $Plg^{-/-}$ mice were located close to the wound margins in all sections examined, and the tips of keratinocyte wedges often had a blunted appearance suggestive of a hindrance in migration (see representative data in Figures 5C and 5D; also see Rømer et al., 1996). By day 17, the wounds from the control, $Fib^{-/-}$, and $Plg^{-/-}/Fib^{-/-}$ mice were well resolved with only a small residual scar at the incision site (see representative data in Figure 5). However, the wounds of $Plg^{-/-}$ mice were sizable with



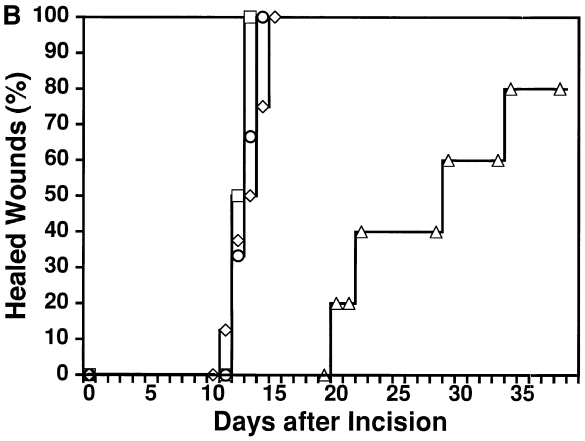


Figure 4. Fib Deficiency Alleviates Impairment of Incisional Skin-Wound Healing in $Plg^{-/-}$ Mice

(A) An example of progress in repair processes in a control mouse, a $Plg^{-/-}$ mouse, a $Fib^{-/-}$ mouse, and a $Plg^{-/-}/Fib^{-/-}$ mouse at days 5, 12, and 16 after surgical incision. At day 5, all wounds are of similar size. The $Fib^{-/-}$ and $Plg^{-/-}/Fib^{-/-}$ wounds appear slightly darker due to accumulation of dried blood at the wound margins. At day 12, a small residual defect is present in the wound of the control, $Fib^{-/-}$, and $Plg^{-/-}/Fib^{-/-}$ mice. In contrast, the wound from the $Plg^{-/-}$ mouse is still large; the scab is lost, and the wound appears red due to protruding granulation tissue. At day 16, the wounds of the control, $Fib^{-/-}$, and $Plg^{-/-}/Fib^{-/-}$ mice have healed, and new fur has grown to cover the wounds. The wound of the $Plg^{-/-}$ mouse is covered by a hard, scaly surface that lacks epidermal coverage.

(B) Plot of the percentage of mice healed versus time after surgical incision: control mice (squares; n = 6), $Plg^{-/-}$ mice (triangles; n = 5), $Fib^{-/-}$ mice (circles; n = 6), and $Plg^{-/-}/Fib^{-/-}$ mice (diamonds; n = 8).

pronounced lingering scabs (Figures 5C and 5D). Despite the obvious slow progress in wound repair in $Plg^{-/-}$ mice, it should be noted that these mice and $Plg^{-/-}/Fib^{-/-}$ and $Fib^{-/-}$ mice uniformly showed many features of normal repair, including formation of granulation tissue and pronounced angiogenesis (Figure 5, and data not shown). However, quantitative differences in these processes cannot presently be excluded.

Discussion

Plg deficiency is compatible with development to term and growth to sexual maturity; however, the pathological consequences of this deficit are severe, and the prospects for long-term survival are bleak (Bugge et al., 1995a; Ploplis et al., 1995; this report). Plg-deficient mice have a myriad of disorders, including progressive and widespread organ damage, wasting, and ultimately premature death. The studies presented here provide direct evidence that fibrin(ogen) is essential to the expression of all spontaneous pathologies previously established in Plg-deficient mice. Furthermore, our results show that one defect revealed in experimentally challenged $Plg^{-/-}$ mice, delayed wound healing in the skin (Rømer et al., 1996), is also effectively corrected by the absence of fibrin(ogen). The latter finding is particularly noteworthy given that wound healing is extremely complex and involves a combination of processes that have been previously tied to Plg activation, including inflammatory response, cell proliferation and migration, angiogenesis, and tissue remodeling (Clark and Henson, 1988; Donaldson and Mahan, 1988; also see the Introduction). Taken together, these findings strongly suggest that the critical and possibly only essential physiological role of the Plg-activation system is fibrinolysis.

Although tissue damage associated with vascular and extravascular fibrin deposits constituted the dominant pathological theme in $Plg^{-/-}$ mice (Bugge et al., 1995a;

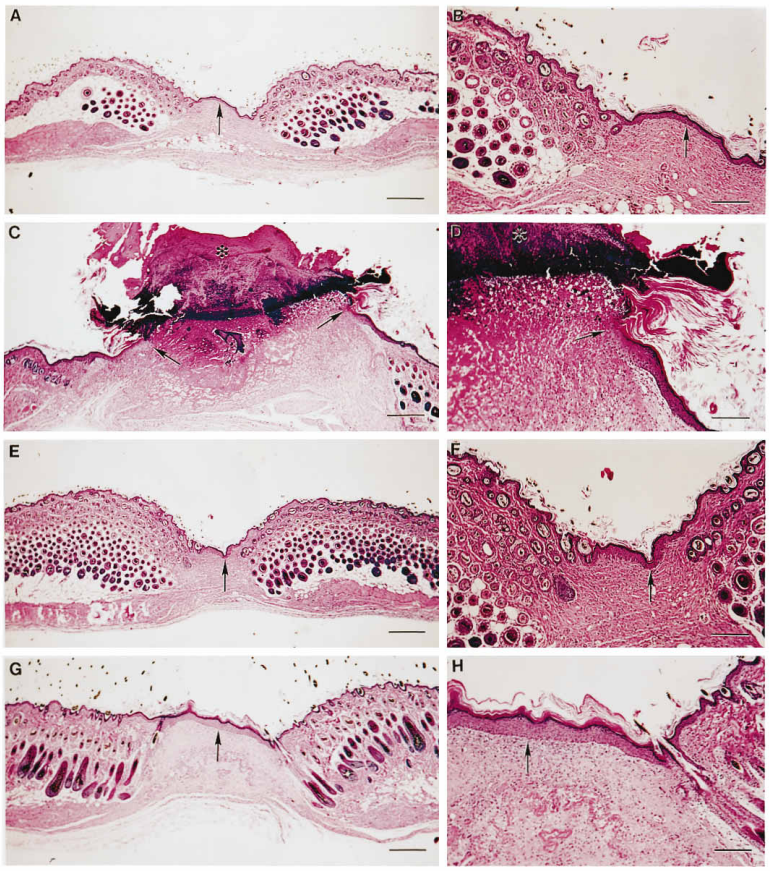


Figure 5. Restoration of Keratinocyte Migration in Plg$^{-/-}$ Mice by Fib Deficiency

Hematoxylin- and eosin-stained histological sections show a representative example of progress in wound repair in a control mouse (A and B), a Plg$^{-/-}$ mouse (C and D), a Fib$^{-/-}$ mouse (E and F), and a Plg$^{-/-}$/Fib$^{-/-}$ mouse (G and H) 17 days after wounding. (A), (C), (E), and (G) are low magnifications showing the overall histological appearance of the wounds. (B), (D), (F), and (H) are high magnifications of the same sections demonstrating the progress in reepithelialization. By day 17, the keratinocyte wedges in the wounds from the control, Fib$^{-/-}$, and Plg$^{-/-}$/Fib$^{-/-}$ mice have met and fused to reepithelialize the wounds completely (arrows). Furthermore, in mice of each of these genotypes, granulation tissue has matured to form a scar. In contrast, at day 17, the wound from the Plg$^{-/-}$ mouse is still covered by a scab (asterisk in C and D), and the keratinocyte wedges (leading edges indicated by arrows) are near the wound margins. Magnification bars in (A), (C), (E), and (G) = 475 μm; (B), (D), (F), and (H) = 200 μm.

Ploplis et al., 1995), a lingering question has been the primary mechanism(s) responsible for spontaneous lesion formation and delayed wound healing in Plg$^{-/-}$ mice. A substantial body of evidence favors a role of Plg outside of fibrinolysis (e.g., general matrix degradation, procollagenase and growth-factor activation), and a failure of Plg to act in nonfibrinolytic processes could initiate lesion formation and secondary local fibrin accumulation. In this regard, it should be noted that the phenotypic rescue of Plg$^{-/-}$ mice by the loss of Fib does not formally exclude either the possibility that Plg participates in processes unrelated to fibrin(ogen) cleavage or the possibility that Plg deficiency initiates spontaneous pathological events in vivo that are unrelated to fibrin(ogen). However, it is clear that any roles of Plg distinctly uncoupled from fibrin(ogen) are either not essential for development, growth to adulthood, efficient repair of skin wounds, and long-term survival, or these biological roles are effectively compensated for by other factors in the absence of Plg. Furthermore, if Plg deficiency in fact initiates fibrin(ogen)-independent pathological events, then these pathologies are either extremely subtle or quickly resolved without the aggravating contribution of an impaired process related to fibrin(ogen) (e.g., fibrinolysis). Of course, the simplest model that can account for the overall good health, longevity, and timely wound healing of Plg$^{-/-}$/Fib$^{-/-}$ mice compared to Plg$^{-/-}$ mice is that the crucial physiological role of Plg in vivo is merely fibrinolysis. Under this model, the initiating event in spontaneous lesion development in Plg$^{-/-}$ mice would be chance or trauma-induced thrombosis and extravascular fibrin deposition. Accumulation and persistence of thrombi and fibrin deposits within tissues in the absence of Plg ultimately results in severe organ damage, impaired tissue repair, and life-threatening illness.

The fact that two Plg activators, uPA and tPA, appear to be an evolutionarily stable feature among mammals argues that these proteins play important, distinct biological roles. However, these unique roles are not necessarily outside of fibrinolysis. The studies presented here, implying that the critical function of Plg is fibrinolysis, reinforce the concept that both physiolologically relevant Plg activators serve in fibrinolysis. This view is also supported by comparative studies of mice with single and combined deficits in uPA, tPA, and uPAR (Carmeliet et al., 1994a; Bugge et al., 1996). Our working hypothesis for the evolutionary advantage of the two distinct Plg activators is that: i) uPA provides a mechanism for efficient cell-mediated fibrinolysis driven by cell-surface binding (uPAR-dependent fibrinolysis) and/or local secretion (receptor-independent fibrinolysis) (Bugge et al., 1996), and ii) tPA provides a mechanism for efficient cell-independent fibrinolysis via its unique fibrin-binding capability (Collen and Lijnen, 1994). Transcriptional regulation of PA, PA inhibitor, and uPAR by cytokines and

other factors (Danø et al., 1985; Saksela and Rifkin, 1988; Vassalli, 1994) and the coupling of uPA-uPAR to cell adhesion (Waltz and Chapman, 1994), signal transduction (Dumler et al., 1993), and protease-clearance systems (Strickland et al., 1995) may modulate or focus cellular fibrinolytic potential in vivo.

A detailed understanding of impaired wound healing in $Plg^{-/-}$ mice will provide valuable insights into the pathophysiological roles of Plg. Impediments in several processes might account for the observed impaired wound healing in $Plg^{-/-}$ mice, including: i) reduction in local growth-factor or matrix-protease activation, ii) failure to generate biologically active fibrin-degradation products, iii) reduction in the ability of responding cells to dissect proteolytically through the fibrin-rich provisional matrices, or iv) some combination of disorders. Although no data is presently available regarding growth-factor and zymogen activation within wound fields in $Plg^{-/-}$ mice, if either of these processes were restricted to the point of slowing tissue repair in $Plg^{-/-}$ mice, then one would not anticipate that the loss of fibrin(ogen) would lift these restrictions or restore prompt wound healing. A connection between production of fibrin-degradation products and faulty wound healing in $Plg^{-/-}$ mice is an intriguing alternative possibility in the light of the substantial body of evidence suggesting that fibrin-degradation products may influence inflammatory and immune responses (Plow and Edgington, 1986), cell proliferation (Robson et al., 1993), angiogenesis (Thompson et al., 1993), chemotaxis (Skogen et al., 1988), and gene expression (Robson et al., 1994). However, once again, if a major restriction on wound healing in $Plg^{-/-}$ mice resulted from loss of biologically active fibrin-degradation products, then one would not expect this obstacle to be lifted if Fib and its derivatives were completely eliminated from the system. Therefore, the proposal that Plg deficiency results in a profound impediment in cellular infiltration into fibrin-rich matrices remains as the simplest hypothesis consistent with phenotypic rescue observed in $Plg^{-/-}/Fib^{-/-}$ mice.

The hypothesis that Plg facilitates cellular penetration of fibrin-containing matrices also fits with earlier results describing the unusual "blunted" morphology of keratinocyte wedges migrating into the wound fields of $Plg^{-/-}$ mice (Rømer et al., 1996). It should be noted that uPA and uPAR expression are induced in migrating keratinocytes within skin wounds (Rømer et al., 1991, 1994). Therefore, local conversion of Plg to plasmin and subsequent fibrin degradation is a likely scenario in normal wound repair. Plasmin is probably one member of a team of carefully regulated and specialized matrix-degrading enzymes, including serine-, metallo-, and other classes of proteases, which together serve in matrix remodeling and cellular reorganization of wound fields. Plasmin may be particularly useful in fibrin solubilization, and without it, cellular reorganization of fibrin-rich matrices is severely impeded. However, despite slow progress in wound repair, wounds in $Plg^{-/-}$ mice eventually resolve with an outcome that is generally comparable to that of control mice. Thus, an interesting unresolved question is what protease(s) contributes to fibrin clearance in the absence of Plg?

Generation of fibrin(ogen)- and Plg-deficient mice shows that dramatic swings in the hemostatic balance in either direction are compatible with life. However, without these factors, life is extremely perilous. On the one hand, fibrin(ogen)-deficient animals are at risk for catastrophic spontaneous or trauma-induced hemorrhagic events, and the challenge of pregnancy is uniformly fatal (Suh et al., 1995). On the other hand, Plg-deficient mice are progressively ravaged by severe thrombosis, cannot effectively resolve wounds, and have a short life expectancy. Although the deleterious consequences of Plg deficiency can be alleviated by the absence of fibrin(ogen), the extraordinary suspension of the hemostatic balance genetically established in $Plg^{-/-}/Fib^{-/-}$ mice is no less precarious than that in $Fib^{-/-}$ mice; like $Fib^{-/-}$ mice, $Plg^{-/-}/Fib^{-/-}$ mice are at risk for life-threatening bleeding and cannot support a pregnancy beyond midgestation. Clearly, an opposing and balanced system of coagulation and fibrinolytic factors is critical to preserve vascular integrity, to maintain free blood flow in the vasculature, and to efficiently repair tissue damage.

If the only essential physiological role of Plg is fibrinolysis, then this does not preclude other important roles of Plg in pathological settings. In the context of pharmacological challenges, microbial pathogens, metabolic disorders, and genetic abnormalities, plasmin might be generated at sites or levels that promote proteolysis of a wider spectrum of substrates than cleaved under normal physiological conditions. For example, production of bacterial Plg activators such as streptokinase and staphylokinase may provide a selective advantage to microorganisms in escaping fibrin-based immobilization and penetrating host tissue barriers. Plg activation may also serve an important general proteolytic role in tumor-cell invasion in vivo (Ossowski and Reich, 1983; Danø et al., 1985, 1994; Mignatti et al., 1986; Ossowski, 1988; Ossowski et al., 1991a, 1991b; Crowley et al., 1993; Kook et al., 1994). Since fibrin appears to be a common component of the tumor stroma (Dvorak, 1986), tumor cells may benefit from plasmin-mediated fibrinolysis in the same sense that migrating keratinocytes appear to benefit when confronting fibrin-rich wound fields. However, tumor cells might also capitalize on the ability of plasmin to activate latent growth factors and proenzymes or degrade general matrix components (Danø et al., 1985, 1994; Saksela and Rifkin, 1988). Any advantage conferred on tumor cells by overexpression of PAs and subsequent local plasmin-mediated proteolysis would presumably be selectively maintained in the context of tumor progression, regardless of whether the plasmin substrates were physiologically relevant. $Plg^{-/-}$, $Fib^{-/-}$, and $Plg^{-/-}/Fib^{-/-}$ mice provide a unique opportunity to explore not only the role of Plg and fibrin(ogen) in tumor progression and metastasis but also to examine the interplay of these factors in disease. Similarly, we expect that these animals will also be a major asset in examining the role of Plg and fibrin(ogen) in atherosclerosis (Plump et al., 1992), sickle-cell disease (Trudel et al., 1994), fibrotic lung disease (Eitzman et al., 1996), bacterial infection (Gyetko et al., 1996), and other diseases that are easily modeled in mice.

Experimental Procedures

Generation of Double-Deficient Mice

$Fib^{-/-}$ and $Fib^{+/-}$ mice of mixed 129/CF-1 background (Suh et al., 1995) were crossed to $Plg^{-/-}$ and $Plg^{+/-}$ mice of mixed 129/Black-Swiss background (Bugge et al., 1995a), followed by interbreeding of the double heterozygous offspring. The genotypes of mice raised were established by multiplex polymerase chain reaction of tail or ear biopsy DNA. The wild-type Fib Aα-chain allele was detected with the primers Fib-ex1–5′ (5′-GCTTCAGCTCCAGTTCTCCTCATG AGCCAT-3′) and Fib-in1–3′ (5′-TGCTGGATCAATCCCCAGCAACCG TGAGAG-3′). These primers generate a 376 bp product. The targeted Fib Aα-chain allele was detected with the primers Fib-ex1–5′ (see above) and HPRT-1 (5′-TATTACCAGTGAATCTTTGTCAGCAG-3′). Together, these primers generate a 283 bp product. The wild-type Plg allele was detected with the primers Plg-in2–3′ (5′-TGTGGGCTC TAAAGATGGAACTCC-3′) and Plg-ex2–5′ (5′-GACAAGGGGACTCG CTGGATGGCTA-3′). These primers generate a 268 bp product. The targeted Plg allele was detected with the primers PGK-HPRT-2 (5′-GTGCGAGGCCAGAGGCCACTTGTGTAGCG-3′) and PLG-in2–3′ (see above). Together, these primers generate a 190 bp product. Mice of all genotypes were housed together in standard facilities with two to four mice per cage and were kept in the same room under supervision by the same investigator throughout the observation period.

Hematological Analysis

Mice were anesthetized with 0.1 ml/30 g body weight ketamine/xylazine/acepromazine (4:1:1), and blood was collected from the inferior vena cava into one-tenth volume of 0.129 M sodium citrate. Blood cell counts and hematocrit were established using a (Technicon H-1) blood-cell analyzer.

Histological Analysis

Mice were sacrificed under ketamine anesthesia (see above). Tissues were either placed immediately into zinc-formalin fixative (U. S. Biotex) or perfused with cold phosphate-buffered saline, followed by perfusion with cold 4% (w/v) paraformaldehyde in phosphate-buffered saline. Fixed tissues were processed into paraffin, sectioned, and stained with hematoxylin and eosin. Histological evaluation of tissue sections was done by an investigator who was blinded to the genotypes of mice. Fibrin(ogen) immunostaining was performed as described previously (Bugge et al., 1995a).

Wound Healing

Young adult mice were anesthetized by inhalation of 2% Isoflurane (Ohmeda PPD, New Jersey) before surgical incision. Full-thickness wounds, 13 mm long, were made in the shaved middorsal skin. The wounds were neither dressed nor sutured. The mice were caged individually, and the degree of wound healing was determined by daily inspection. Surgery and evaluation of macroscopic progress of wound healing was done by an investigator blinded to the genotypes of the mice. Wound tissues were collected following perfusion fixation of mice with 4% paraformaldehyde as described above. Paraffin-embedded sections were cut perpendicular to the original wound incisions, stained with hematoxylin and eosin, and evaluated by a blinded investigator. Animal care at the Division of Veterinary Services, Children's Hospital Research Foundation was in accordance with the American Association for Accreditation of Laboratory Animal Care.

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